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(54) Title: MUTANT α-AMYLASE HAVING INTRODUCED THEREIN A DISULFIDE BOND

(57) Abstract

Novel α-amylase enzymes are disclosed in which one or more disulfide bonds are introduced into the enzyme via addition or substitution of a residue with a cysteine. The disclosed α-amylase enzymes show altered or improved stability and/or activity profiles.

MUTANT α-AMYLASE
HAVING INTRODUCED THEREIN A DISULFIDE BOND

FIELD OF THE INVENTION

5   The present invention is directed to mutant α-amylases having introduced therein one or more disulfide bonds. In particular, the disulfide bonds are introduce by mutation of a precursor α-amylase to introduce one or more cysteine residues so as to produce a disulfide bond between two cysteine residues in said mutant α-amylase. It is specifically contemplated that the mutant will have altered performance characteristics such as altered stability and/or altered activity profiles.

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BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) hydrolyze internal α-1,4-glucosidic linkages in starch, largely at random, to produce smaller molecular weight malto-dextrins. α-Amylases are of considerable commercial value, being used in the initial stages
15   (liquefaction) of starch processing; in alcohol production; as cleaning agents in detergent matrices; and in the textile industry for starch desizing. α-Amylases are produced by a wide variety of microorganisms including Bacillus and Aspergillus, with most commercial amylases being produced from bacterial sources such as Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus subtilis, or Bacillus stearothermophilus. In recent years, the preferred enzymes in commercial use
20   have been those from Bacillus licheniformis because of their heat stability and performance under commercial operating conditions.

In general, starch to fructose processing consists of four steps: liquefaction of granular starch, saccharification of the liquefied starch into dextrose, purification, and isomerization to fructose. The object of a starch liquefaction process is to convert a concentrated suspension of
25   starch polymer granules into a solution of soluble shorter chain length dextrins of low viscosity. This step is essential for convenient handling with standard equipment and for efficient conversion to glucose or other sugars. To liquefy granular starch, it is necessary to gelatinize the granules by raising the temperature of the granular starch to over about 72°C. The heating process instantaneously disrupts the insoluble starch granules to produce a water soluble starch solution.
30   The solubilized starch solution is then liquefied by α-amylase (EC 3.2.1.1.).

A common enzymatic liquefaction process involves adjusting the pH of a granular starch slurry to between 6.0 and 6.5, the pH optimum of α-amylase derived from Bacillus licheniformis, with the addition of calcium hydroxide, sodium hydroxide or sodium carbonate. The addition of calcium hydroxide has the advantage of also providing calcium ions which are known to stabilize the α-
35   amylases against inactivation. Upon addition of α-amylases , the suspension is pumped through a steam jet to instantaneously raise the temperature to between 80-115°C. The starch is immediately

gelatinized and, due to the presence of α-amylases, depolymerized through random hydrolysis of a(1-4) glycosidic bonds to a fluid mass which is easily pumped.

In a second variation to the liquefaction process, α-amylase is added to the starch suspension, the suspension is held at a temperature of 80-100°C to partially hydrolyze the starch

5 granules, and the partially hydrolyzed starch suspension is pumped through a jet at temperatures in excess of about 105°C to thoroughly gelatinize any remaining granular structure. After cooling the gelatinized starch, a second addition of α-amylase can be made to further hydrolyze the starch.

A third variation of this process is called the dry milling process. In dry milling, whole grain is ground and combined with water. The germ is optionally removed by flotation separation or

10 equivalent techniques. The resulting mixture, which contains starch, fiber, protein and other components of the grain, is liquefied using α-amylase. The general practice in the art is to undertake enzymatic liquefaction at a lower temperature when using the dry milling process. Generally, low temperature liquefaction is believed to be less efficient than high temperature liquefaction in converting starch to soluble dextrins.

15 Typically, after gelatinization the starch solution is held at an elevated temperature in the presence of α-amylase until a DE of 10-20 is achieved, usually a period of 1-3 hours. Dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100.

20 The maximum temperature at which the starch solution containing α-amylase can be held depends upon the microbial source from which the enzyme was obtained and the molecular structure of the α-amylase molecule. α-Amylases produced by wild type strains of Bacillus subtilis or Bacillus amyloliquefaciens are typically used at temperatures no greater than about 90°C due to excessively rapid thermal inactivation above that temperature, whereas α-amylases produced by

25 wild type strains of Bacillus licheniformis can be used at temperatures up to about 110°C. The presence of starch and calcium ion are known to stabilize α-amylases against inactivation. Nonetheless, α-amylases are used at pH values above 6 to protect against rapid inactivation. At low temperatures, α-amylase from Bacillus licheniformis is known to display hydrolyzing activity on starch substrate at pH values as low as 5. However, when the enzyme is used for starch

30 hydrolysis at common jet temperatures, e.g., between 102°C and 109°C, the pH must be maintained above at least pH 5.7 to avoid excessively rapid inactivation. The pH requirement unfortunately provides a narrow window of processing opportunity because pH values above 6.0 result in undesirable by-products, e.g., maltulose. Therefore, in reality, liquefaction pH is generally maintained between 5.9 and 6.0 to attain a satisfactory yield of hydrolyzed starch.

35 Another problem relating to pH of liquefaction is the need to raise the pH of the starch suspension from about 4, the pH of a corn starch suspension as it comes from the wet milling stage, to 5.9-6.0. This pH adjustment requires the costly addition of acid neutralizing chemicals

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and also requires additional ion-exchange refining of the final starch conversion product to remove the chemical. Moreover, the next process step after liquefaction, typically saccharification of the liquefied starch into glucose with glucoamylase, requires a pH of 4-4.5; therefore, the pH must be adjusted back down from 5.9-6.0 to 4-4.5; requiring additional chemical addition and refining steps.

5   Subsequent to liquefaction, the processed starch is saccharified to glucose with glucoamylase. A problem with present processes occurs when residual starch is present in the saccharification mixture due to an incomplete liquefaction of the starch, e.g., inefficient amylose hydrolysis by amylase. Residual starch is highly resistant to glucoamylase hydrolysis. It represents a yield loss and interferes with downstream filtration of the syrups.

10   Additionally, many α-amylases are known to require the addition of calcium ion for stability. This further increases the cost of liquefaction.

In U.S. Patent No. 5,322,778, liquefaction between pH 4.0 and 6.0 was achieved by adding an antioxidant such as bisulfite or a salt thereof, ascorbic acid or a salt thereof, erythorbic acid, or phenolic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, or a-tocopherol

15   to the liquefaction slurry. According to this patent, sodium bisulfite must be added in a concentration of greater than 5mM.

In U.S. Patent No. 5,180,669, liquefaction between a pH of 5.0 to 6.0 was achieved by the addition of carbonate ion in excess of the amount needed to buffer the solution to the ground starch slurry. Due to an increased pH effect which occurs with addition of carbonate ion, the slurry is

20   generally neutralized by adding a source of hydrogen ion, for example, an inorganic acid such as hydrochloric acid or sulfuric acid.

In PCT Publication No. WO 95/35382, a mutant α-amylase is described having improved oxidation stability and having changes at positions 104, 128, 187 and/or 188 in *B. licheniformis* α-amylase.

25   In PCT Publication No. WO 96/23873, mutant α-amylases are described which have any of a number of mutations.

In PCT Publication No. WO 94/02597, a mutant α-amylase having improved oxidative stability is described wherein one or more methionines are replaced by any amino acid except cysteine or methionine.

30   In PCT publication No. WO 94/18314, a mutant α-amylase having improved oxidative stability is described wherein one or more of the methionine, tryptophan, cysteine, histidine or tyrosine residues is replaced with a non-oxidizable amino acid.

In PCT Publication No. WO 91/00353, the performance characteristics and problems associated with liquefaction with wild type *Bacillus licheniformis* α-amylase are approached by

35   genetically engineering the α-amylase to include the specific substitutions Ala-111-Thr, His-133-Tyr and/or Thr-149-Ile.

Studies using recombinant DNA techniques to explore which residues are important for the catalytic activity of amylases and/or to explore the effect of modifying certain amino acids within the active site of various amylases and glycosylases have been conducted by various researchers (Vihinen et al., J. Biochem., Vol. 107, pp. 267-272 (1990); Holm et al., Protein Engineering, Vol. 3,

5   pp. 181-191 (1990); Takase et al., Biochemica et Biophysica Acta, Vol. 1120, pp. 281-288 (1992); Matsui et al., FEBS Letters, Vol. 310, pp. 216-218 (1992); Matsui et al., Biochemistry, Vol. 33, pp. 451-458 (1992); Sogaard et al., J. Biol. Chem., Vol. 268, pp. 22480-22484 (1993); Sogaard et al., Carbohydrate Polymers, Vol. 21, pp. 137-146 (1993); Svensson, Plant Mol. Biol., Vol. 25, pp. 141-157 (1994); Svensson et al., J. Biotech. Vol. 29, pp. 1-37 (1993)). Researchers have also studied

10 which residues are important for thermal stability (Suzuki et al., J. Biol. Chem., Vol. 264, pp. 18933-18938 (1989); Watanabe et al., Eur. J. Biochem. Vol. 226, pp. 277-283 (1994)); and one group has used such methods to introduce mutations at various histidine residues in a *Bacillus licheniformis* amylase, the rationale being that *Bacillus licheniformis* amylase which is known to be relatively thermostable when compared to other similar *Bacillus* amylases, has an excess of

15 histidines and, therefore, it was suggested that replacing a histidine could affect the thermostability of the enzyme. This work resulted in the identification of stabilizing mutations at the histidine residue at the +133 position and the alanine residue at position +209 (Declerck et al., J. Biol. Chem., Vol. 265, pp. 15481-15488 (1990); FR 2 665 178-A1; Joyet et al., Bio/Technology, Vol. 10, pp. 1579-1583 (1992)).

20   The introduction of di-sulphide bonds into proteins by site-directed mutagenesis affords a means of stabilizing native, folded conformations, see e.g., Villafranca et al., Science, Vol. 222, pp. 782-788 (1983). Hazes et al., Prot. Eng., Vol. 2, No. 2, pp. 119-125 (1988) suggest introducing disulfide bonds to a protein via a modeling algorithm which starts with the generation of the Cβ position from the N, Cα and C atom positions available from a known three-dimensional model. A

25 first set of residues is selected on the basis of the Cβ-Cβ distances; the Sγ positions are generated which satisfy the requirement that, with ideal values for the Cα-Cβ and Cβ-Sγ bond lengths and for the bond angle at Cβ, the distance between the Sγ of residue 1 and Cβ of residue 2 in a pair (determined by the bond angle at Sγ2) is at, or very close to, its ideal value; and the two acceptable Sγ positions are found for each cysteine and the four different conformations for each disulfide

30 bond established. Finally the four conformations are subjected to energy minimization procedure to remove large deviations from ideal geometry and their final energies calculated. Sowdhamini et al., Prot. Eng., Vol. 3, No. 2, pp. 95-103 (1989) discloses that the introduction of disulfide bonds into proteins by site directed mutagenesis affords a means of stabilizing native folded conformations and suggests computer modeling techniques for assessing the stereochemical

35 suitability of pairs of residues in proteins as potential sites for introduction of cysteine disulfide crosslinks. The authors suggest that the elemental condition for considering residue positions in proteins, as potential sites for cysteine introduction, to generate unstrained disulfides is that the

alpha carbon distance between the two cysteine residues to be joined via the disulfide bond ($C\alpha$-$C\alpha$) be less than or equal to 6.5 Angstroms and that the beta carbon distance between the two cysteine residues to be joined via the disulfide bond ($C\beta$-$C\beta$) distance of less than or equal to 4.5 Angstroms.

Despite the advances made in the prior art, a need exists for an α-amylase which is more effective in commercial liquefaction processes but which allows activity at lower pH than currently practical. Additionally, a need exists for improved amylases having characteristics which makes them more effective under the conditions of detergent use. Because commercially available amylases are not acceptable under many conditions due to stability problems, for example, the high alkalinity and oxidant (bleach) levels associated with detergents, or temperatures under which they operate, there is a need for an amylase having altered, and preferably increased, performance profiles under such conditions.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an α-amylase having altered performance profiles.

It is a further object of the present invention to provide an α-amylase having improved stability at high temperature.

Accordingly, the present invention provides a mutant α-amylase having introduced therein one or more cysteine residues, wherein at least one of the introduced cysteine residues is capable of forming a disulfide bond with another cysteine residue. Preferably, the introduced cysteine(s) and the other cysteine residue with which it is to form a disulfide bond correspond to positions in the precursor α-amylase having a $C\alpha$-$C\alpha$ bond distance of between about 4.4-6.8 Angstroms and a $C\beta$-$C\beta$ bond distance of between about 3.45 and 4.5 Angstroms. In a particularly preferred embodiment of the invention, the α-amylase is derived from a bacterial or a fungal source and comprises a substitution corresponding to E119C/S130C and/or D124C/R127C *Bacillus licheniformis*. Most preferably, the α-amylase is derived from *Bacillus*.

The invention further comprises nucleic acids encoding such mutant amylases, vectors comprising such nucleic acids, host cells transformed with such vectors and methods of expressing mutant α-amylases utilizing such host cells.

The invention further comprises the use of the mutant α-amylases according to the invention to liquefy starch in the starch processing pathway to glucose or other starch derivatives, as an additive in detergents such as laundry and dishwashing detergents, as a baking aid and for desizing of textiles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the regions of secondary structure which are stabilized by the introduction of E119C/S130C and D124C/R127C corresponding to *Bacillus licheniformis* α-amylase.

5   Figure 2 illustrates the DNA sequence of the gene for α-amylase from *Bacillus licheniformis* (NCIB 8061) (SEQ ID NO:1) and deduced amino acid sequence of the translation product (SEQ ID NO:2) as described by Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986).

Figure 3 illustrates the amino acid sequence (SEQ ID NO:3) of the mature α-amylase enzyme from *Bacillus licheniformis*.

10   Figure 4 illustrates an alignment of the primary structures of three *Bacillus* α-amylases. The *Bacillus licheniformis* α-amylase (Am-Lich) (SEQ ID NO:4) is described by Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986); the *Bacillus amyloliquefaciens* α-amylase (Am-Amylo) (SEQ ID NO:5) is described by Takkinen et al., J. Biol. Chem., Vol. 258, pp. 1007-1013 (1983); and the *Bacillus stearothermophilus* α-amylase (Am-Stearo) (SEQ ID NO:6) is described by Ihara et al.,

15   J. Biochem., Vol. 98, pp. 95-103 (1985).

Figure 5 illustrates plasmid pHP13 wherein Cm^R refers to chloramphenicol resistance, Em^R refers to erythromycin resistance and Rep pTA1060 refers to the origin of replication from plasmid pTA1060.

Figure 6 illustrates the pBLapr plasmid wherein BL AA refers to *Bacillus licheniformis* α-

20   amylase gene; *aprE* refers to the promoter and signal peptide encoding region of the *aprE* gene; AmpR refers to the ampicillin resistant gene from pBR322; and CAT refers to the chloramphenicol resistance gene from pC194.

Figure 7 illustrates the pHP.BL plasmid carrying the gene for *Bacillus licheniformis* α-amylase.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

"α-Amylase" means an enzymatic activity which cleaves or hydrolyzes the α(1-4)glycosidic bond, e.g., that in starch, amylopectin or amylose polymers. α-Amylase as used herein includes naturally occurring α-amylases as well as recombinant α-amylases. Preferred α-amylases in the

30   present invention are those derived from *Bacillus licheniformis, Bacillus amyloliquefaciens* or *Bacillus stearothermophilus*, as well as fungal α-amylases such as those derived from *Aspergillus* (i.e., *A. oryzae* and *A. niger*).

"Recombinant α-amylase" means an α-amylase in which the DNA sequence encoding the naturally occurring α-amylase is modified to produce a mutant DNA sequence which encodes the

35   substitution, insertion or deletion of one or more amino acids in the α-amylase sequence compared to the naturally occurring α-amylase.

"Expression vector" means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome-binding sites, and sequences which control termination of transcription and translation. A preferred promoter is the *Bacillus subtilis aprE* promoter. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, plasmid and vector are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

"Host strain" or "host cell" means a suitable host for an expression vector comprising DNA encoding the α-amylase according to the present invention. Host cells useful in the present invention are generally procaryotic or eucaryotic hosts, including any transformable microorganism in which the expression of α-amylase according to the present invention can be achieved. Specifically, host strains of the same species or genus from which the α-amylase is derived are suitable, such as a *Bacillus* strain. Preferably, an α-amylase negative *Bacillus* strain (genes deleted) and/or an α-amylase and protease deleted *Bacillus* strain (ΔamyE, Δapr, Δnpr) is used. Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the α-amylase and its variants (mutants) or expressing the desired α-amylase.

"Liquefaction" or "liquefy" means a process by which starch is converted to shorter chain and less viscous dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of α-amylase.

According to the present invention, a mutant α-amylase is provided that has introduced therein a first cysteine residue which is capable of forming a disulfide bond with a second cysteine residue. Preferably, the first cysteine residue comprises an addition or a substitution to a precursor α-amylase. It is further possible to incorporate the second cysteine residue as an addition or substitution as well, and this may be preferable should a useful cysteine residue not be present in a location useful to stabilize the desired portion of the molecule. With respect to *Bacillus licheniformis* α-amylase, it is necessary to incorporate two cysteine residues as the wild type molecule possesses no cysteines. Addition or substitution of an amino acid as used herein refers to any modification of the amino acid sequence itself of the precursor α-amylase, but preferably refers to using genetic engineering to mutate a nucleic acid encoding the precursor α-amylase so as to encode the substituted or added cysteine residue in the expressed protein. The precursor α-amylases include naturally occurring α-amylases and recombinant α-amylases. Modification of the

precursor DNA sequence which encodes the amino acid sequence of the precursor α-amylase can be by methods described herein and in commonly owned U.S. Patent Nos. 4,760,025 and 5,185,258, incorporated herein by reference.

Also provided is a nucleic acid molecule (DNA) which encodes an amino acid sequence
5   comprising the mutant α-amylase provided by the present invention, expression systems incorporating such DNA including vectors and phages, host cells transformed with such DNA, and anti-sense strands of DNA corresponding to the DNA molecule which encodes the amino acid sequence. Similarly, the present invention includes a method for producing a mutant α-amylase by expressing the DNA incorporated on an expression system which has been transformed into a
10   host cell. The mutant α-amylase of the invention may be used in liquefaction of starch, as an ingredient in laundry detergents, automatic dishwashing detergents, hard surface cleaning products, in food processing including baking applications, in textile processing including as a desize agent, or in any other application in which α-amylase activity is useful.

The precursor α-amylase is produced by any source capable of producing α-amylase.
15   Suitable sources of α-amylases are prokaryotic or eukaryotic organisms, including fungi, bacteria, plants or animals. Preferably, the precursor α-amylase is produced by a *Bacillus*; more preferably, by *Bacillus licheniformis, Bacillus amyloliquefaciens* or *Bacillus stearothermophilus*; most preferably, the precursor α-amylase is derived from *Bacillus licheniformis*.

Homologies have been found between almost all endo-amylases sequenced to date,
20   ranging from plants, mammals, and bacteria (Nakajima et al., Appl. Microbiol. Biotechnol., Vol. 23, pp. 355-360 (1986); Rogers, Biochem. Biophys. Res. Commun., Vol. 128, pp. 470-476 (1985); Janecek, Eur. J. Biochem., Vol. 224, pp. 519-524 (1994)). There are four areas of particularly high homology in certain *Bacillus* amylases, as shown in Figure 4, wherein the underlined sections designate the areas of high homology. Sequence alignments have also been used to map the
25   relationship between *Bacillus* endo-amylases (Feng et al., J. Molec. Evol., Vol. 35, pp. 351-360 (1987)). The relative sequence homology between *Bacillus stearothermophilus* and *Bacillus licheniformis* amylase is about 66% and that between *Bacillus licheniformis* and *Bacillus amyloliquefaciens* amylases is about 81%, as determined by Holm et al., Protein Engineering, Vol. 3, No. 3, pp. 181-191 (1990). While sequence homology is important, it is generally recognized
30   that structural homology is also important in comparing amylases or other enzymes. For example, structural homology between fungal amylases and bacterial amylase has been suggested and, therefore, fungal amylases are encompassed within the present invention.

Among others, substitutions at residues corresponding to E119C/S130C and/or D124C/R127C in *Bacillus licheniformis* α-amylase are identified herein for substitution. Thus,
35   specific residues such as E119 refer to an amino acid position number (i.e., +119) which references the number assigned to the mature *Bacillus licheniformis* α-amylase sequence illustrated in Figure 2. The invention, however, is not limited to the mutation of the particular

mature α-amylase of *Bacillus licheniformis* but extends to precursor α-amylases containing amino acid residues at positions which are equivalent to the particular identified residue in *Bacillus licheniformis* α-amylase. A residue of a precursor α-amylase is equivalent to a residue of *Bacillus licheniformis* α-amylase if it is either homologous (i.e., corresponds in position for either the primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus licheniformis* α-amylase (i.e., having the same or similar functional capacity to combine, react, or interact chemically or structurally).

In order to establish homology to primary structure, the amino acid sequence of a precursor α-amylase is directly compared to the *Bacillus licheniformis* α-amylase primary sequence and particularly to a set of residues known to be invariant to all α-amylases for which sequences are known (see e.g., Figure 4). It is possible also to determine equivalent residues by tertiary structure analysis of the crystal structures reported for porcine pancreatic α-amylase (Buisson et al., EMBO Journal, Vol. 6, pp. 3909-3916 (1987); Qian et al., Biochemistry, Vol. 33, pp. 6284-6294 (1994); Larson et al., J. Mol. Biol., Vol. 235, pp. 1560-1584 (1994)); Taka-amylase A from *Aspergillus oryzae* (Matsuura et al., J. Biochem. (Tokyo), Vol. 95, pp. 697-702 (1984)); and an acid α-amylase from *A. niger* (Boel et al., Biochemistry, Vol. 29, pp. 6244-6249 (1990)), with the former two structures being similar, and for barley α-amylase (Vallee et al., J. Mol. Biol., Vol. 236, pp. 368-371(1994); Kadziola, J. Mol. Biol., Vol. 239, pp. 104-121 (1994)). Several preliminary studies have been published related to the secondary structure of α-amylase, i.e., (Suzuki et al., J. Biochem., Vol. 108, pp. 379-381 (1990); Lee et al., Arch. Biochem. Biophys, Vol. 291, pp. 255-257 (1991); Chang et al., J. Mol. Biol., Vol. 229, pp. 235-238 (1993); Mizuno et al., J. Mol. Biol., Vol. 234, pp. 1282-1283 (1993)), and at least one structure has been published for crystalline *Bacillus licheniformis* α-amylase (Machius et al., J. Mol. Biol. Vol. 246, pp. 545-549 (1995)). However, several researchers have predicted common super-secondary structures between glucanases (MacGregor et al., Biochem. J., Vol. 259, pp. 145-152 (1989)) and within α-amylases and other starch-metabolising enzymes (Jaspersen, J. Prot. Chem. Vol. 12, pp. 791-805 (1993); MacGregor, Starke, Vol. 45, pp. 232-237 (1993)); and sequence similarities between enzymes with similar super-secondary structures to α-amylases (Janecek, FEBS Letters, Vol. 316, pp. 23-26 (1993); Janecek et al., J. Prot. Chem., Vol. 12, pp. 509-514 (1993)). A structure for the *Bacillus stearothermophilus* enzyme has been modeled on that of Taka-amylase A (Holm et al., Protein Engineering, Vol. 3, pp. 181-191 (1990)). The four highly conserved regions shown in Figure 4 contain many residues thought to be part of the active-site (Matsuura et al., J. Biochem. (Tokyo), Vol. 95, pp. 697-702 (1984); Buisson et al., EMBO Journal, Vol. 6, pp. 3909-3916 (1987); Vihinen et al., J. Biochem., Vol. 107, pp. 267-272 (1990)) including His +105; Arg +229; Asp +231; His +235; Glu +261 and Asp +328 under the *Bacillus licheniformis* numbering system.

In the practice of the present invention, certain parameters are useful to determine with specificity appropriate substitutions. Information regarding the crystal structure of a specific enzyme should be obtained. Crystal structures from *Bacillus licheniformis* α-amylase, pig pancreatic α-amylase, *Aspergillus niger* and *Aspergillus oryzae* α-amylase, and barley α-amylase

5   indicated, supra, are useful for this purpose. With information related to the crystal structure of the target enzyme, it is then useful to obtain information related to the instability of the enzyme under specific conditions, and preferably to obtain information related to the presence of specific unstable residues or regions which contribute to overall instability of the enzyme. As a specific example, Applicants were aware that α-amylase derived from *Bacillus licheniformis* comprises several

10   residues that are particularly unstable under oxidizing conditions, i.e., M197 and W138. In the practice of the present invention, Applicants specifically studied the region surrounding S148 due to the ability of the S148N mutation to confer increased stability. The hypothesis that this region may have secondary structure elements which are made more stable by this substitution led Applicants to attempt to stabilize the enzyme via introduction of disulfide bonds into the proximal

15   regions of secondary structure.

To determine the specific residues for substitution, the distances between the alpha carbons and the beta carbons for each of the residues in the folded protein were measured from the crystal structure to determine which pairs fall within the allowable distances for a disulfide bond were both residues cysteines. For example, the residue pair for which it is contemplated to

20   incorporate substitution(s) resulting in two cysteine residues should preferably have an alpha carbon distance (Cα-Cα) of between about 4.4 and about 6.8 Angstroms. Similarly, the beta carbon distance (Cβ-Cβ) should be between about 3.45 and 4.5 Angstroms. Selecting amino acid pairs which fall within these criteria for carbon distance and utilizing strategies outlined in Swodhamini et al., supra, and Hazes et al., supra, it was possible to minimize the disturbance of

25   the protein which may be caused by the substitution of a cysteine and the subsequent formation of a disulfide bond between two such cysteines. In this way, it is possible to select a first and second cysteine residue for which conditions are favorable for the formation of a disulfide bond. Applicants thus singled out D124-R127 and E119-S130 as particularly preferred substitutions. However, any residue pair may be utilized so long as it falls within the appropriate criteria provided

30   herein.

α-Amylases according to the present invention which exhibit altered performance characteristics providing desirable and unexpected results are useful in the various applications for which α-amylases are commonly used. For example, α-amylases according to the present invention which exhibit altered performance characteristics at low pH, including improved

35   thermostability, improved pH stability and/or improved oxidative stability, are useful in low pH liquefaction of starch. Enhanced thermostability will be useful in extending the shelf life of products which incorporate them. Enhanced oxidative stability or improved performance is

particularly desirable in cleaning products, and for extending the shelf life of α-amylase in the presence of bleach, perborate, percarbonate or peracids used in such cleaning products. To the contrary, reduced thermal stability or oxidative stability may be useful in industrial processes which require the rapid and efficient quenching of amylolytic activity.

5   α-Amylases of the present invention which exhibit improved low pH stability will be especially useful in starch processing and particularly in starch liquefaction. Conditions present during commercially desirable liquefaction processes characteristically include low pH, high temperature and potential oxidation conditions requiring α-amylases exhibiting improved low pH performance, improved thermal stability and improved oxidative stability. Accordingly, α-amylases

10   according to the present invention which are particularly useful in liquefaction exhibit improved performance at a pH of less than about 6, preferably less than about 5.5, and most preferably less than about 5.0. Additionally, α-amylases according to the present invention which exhibit increased thermal stability at temperatures of between about 80-120°C, and preferably between about 100-110°C, and increased stability in the presence of oxidants will be particularly useful.

15   Additional components known by those skilled in the art to be useful in liquefaction, including, for example, antioxidants, calcium, ions, salts or other enzymes such as endoglycosidases, cellulases, proteases, lipases or other amylase enzymes may be added depending on the intended reaction conditions. For example, combinations of the α-amylase according to the present invention with α-amylases from other sources may provide unique action

20   profiles which find particular use under specific liquefaction conditions. In particular, it is contemplated that the combination of the α-amylase according to the present invention with α-amylase derived from *Bacillus stearothermophilus* will provide enhanced liquefaction at pH values below 5.5 due to complementary action patterns.

      During liquefaction, starch, specifically granular starch slurries from either a wet or dry

25   milled process, is treated with an α-amylase of the present invention according to known liquefaction techniques. Generally, in the first step of the starch degradation process, the starch slurry is gelatinized by heating at a relatively high temperature (between about 80°C and about 110°C). After the starch slurry is gelatinized, it is liquefied using an α-amylase.

      In another embodiment of the present invention, detergent compositions in either liquid, gel

30   or granular form, which comprise the α-amylase according to the present invention may be useful. Such detergent compositions will particularly benefit from the addition of an α-amylase according to the present invention which has increased thermal stability to improve shelf-life or increased oxidative stability such that the α-amylase has improved resistance to bleach or peracid compounds commonly present in detergents. Thus, α-amylase according to the present invention

35   may be advantageously formulated into known powdered, liquid or gel detergents having a pH of between about 6.5 and about 12.0. Detergent compositions comprising the α-amylase according to the present invention may further include other enzymes such as endoglycosidases, cellulases,

proteases, lipases or other amylase enzymes, particularly α-amylase derived from *Bacillus stearothermophilus,* as well as additional ingredients as generally known in the art.

A preferred embodiment of the present invention further comprises, in addition to the substitution of two or more cysteine residues, any one or more of the substitutions known in the art

5   to confer stability or increased activity. For example, the deletion or substitution of a methionine residue or a tryptophan residue, for example M15, M197 or W138 as described in WO 94/18314, the disclosure of which is incorporated herein by reference; substitution at H133Y as described in PCT Publication No. WO 91/00353; or substitution at A209 as described in DeClerck, et al., J. Biol. Chem., Vol. 265, pp. 15481-15488 (1990); or any of the substitutions described in PCT Publication

10   Nos.

WO 95/10603, WO 96/23873 and WO 96/23874. In particularly preferred embodiments, the α-amylase according to the present invention may further comprise a deletion or substitution at one or more residues corresponding to M15, A33, A52, S85, N96, V128, H133, S148N, S187, N188, A209, A269 and/or A379 in *Bacillus licheniformis* α-amylase. Particular embodiments of the

15   amylase of the present invention may comprise a substitution pattern corresponding to M15T/E119C/S130C/N188S, M15L/E119C/S130C/N188S, M15T/E119C/S130C/H133Y/N188S, M15T/E119C/S130C/H133Y/N188S/A209V, M15T/E119C/S130C/N188S/A209V, M15T/E119C/V128E/S130C/H133Y/N188S, M15T/E119C/S130C/S187D/N188S, M15T/E119C/S130C/H133Y, M15T/E119C/S130C/H133Y/N188S/A209V,

20   M15T/E119C/S130C/H133Y/A209V or, M15T/E119C/S130C/H133Y/S148N/A209V/A379S in *Bacillus licheniformis.*

Embodiments of the present invention which comprise a combination of the α-amylase according to the present invention with protease enzymes preferably include oxidatively stable proteases such as those described in U.S. Re. 34,606, incorporated herein by reference, as well as

25   commercially available enzymes such as DURAZYM (Novo Nordisk) and PURAFECT® OxP (Genencor International, Inc.). Methods for making such protease mutants (oxidatively stable proteases), and particularly such mutants having a substitution for the methionine at a position equivalent to M222 in *Bacillus amyloliquefaciens,* are described in U.S. Re. 34,606.

An additional embodiment of the present invention comprises DNA encoding an α-amylase

30   according to the present invention and expression vectors comprising such DNA. The DNA sequences may be expressed by operably linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate host according to well-known techniques. A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors,

35   for example, include segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as the various known plasmids and phages useful for this purpose. In addition, any of a wide variety of expression control sequences are generally used in these vectors. For example,

Applicants have discovered that a preferred expression control sequence for *Bacillus* transformants is the *aprE* signal peptide derived from *Bacillus subtilis*.

A wide variety of host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well-known eukaryotic and prokaryotic hosts, such as strains

5 of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, various fungi, yeast and animal cells. Preferably, the host expresses the α-amylase of the present invention extracellularly to facilitate purification and downstream processing. Expression and purification of the mutant α-amylase of the invention may be effected through art-recognized means for carrying out such processes.

The improved α-amylases according to the present invention are contemplated to provide

10 several important advantages when compared to wild type *Bacillus* α-amylases. For example, one advantage is the increased activity found at low pH and high temperatures typical of common starch liquefaction methods. Another advantage is the increased high pH and oxidative stability which facilitates their use in detergents. Another advantage is that a more complete hydrolysis of starch molecules is achieved which reduces residual starch in the processing stream. Yet another

15 advantage is their improved stability in the absence of calcium ion. Yet another advantage is that the addition of equal protein doses of α-amylase according to the invention provide superior performance when compared to wild type *Bacillus licheniformis* α-amylase due to improvements in both specific activity and stability under stressed conditions. In other words, because of the generally increased stability of the amylases according to the present invention, the increased

20 specific activity on starch of the inventive amylases translates to even greater potential performance benefits of this variant. Under conditions where the wild type enzyme is being inactivated, not only does more of the inventive amylase survive because of its increased stability, but also that which does survive expresses proportionally more activity because of its increased specific activity.

25 The following is presented by way of example and is not to be construed as a limitation to the scope of the claims. Abbreviations used herein, particularly three letter or one letter notations for amino acids are described in Dale, J.W., Molecular Genetics of Bacteria, John Wiley & Sons, (1989) Appendix B.

EXAMPLES

30 ### EXAMPLE 1
Construction Of Plasmid pHP.BL

The α-amylase gene shown in Figure 2 was cloned from *Bacillus licheniformis* NCIB8061 (Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986)). The 1.72kb PstI-SstI fragment,

35 encoding the last three residues of the signal sequence, the entire mature protein and the terminator region, was subcloned into M13mp18. A synthetic terminator was added between the BclI and SstI sites using a synthetic oligonucleotide cassette of the form:

5'-GATCAAAACATAAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTGAGCT-3' (SEQ ID NO:7)

3'-TTTTGTATTTTTTGGCCGGAACCGGGGCGGCCAAAAAAATAATAAAAAC-5' (SEQ ID NO:8)

designed to contain the *Bacillus amyloliquefaciens* subtilisin transcriptional terminator (Wells et al.,

5   Nucleic Acid Research, Vol. 11, pp. 7911-7925 (1983)).

The pBLapr plasmid was constructed carrying the gene for the *Bacillus licheniformis* α-amylase. As illustrated in Figure 6, pBLapr comprises a 6.1kb plasmid including the ampicillin resistance gene from pBR322 and the chloramphenicol resistance gene from pC194, the aprE promoter and the gene encoding for the *Bacillus licheniformis* α-amylase ("BL AA"). The aprE

10   promoter was constructed from a 660bp HindIII-PstI fragment encoding for the promoter and signal sequence of the *Bacillus subtilis* alkaline protease. The PstI site was removed, and an SfiI site added close to the *aprE*/BL AA junction. The BL AA gene comprises the 1720 bp PstI-SstI fragment described above. In the work described herein, pBLapr was constructed with an SfiI site adjacent to the 5' end of the start of the coding sequence for the mature amylase gene.

15   Specifically, the 5' end of the pBLapr construction was subcloned on an EcoRI-SstII fragment from pBLapr into M13BM20 (Boehringer Mannheim) to obtain a coding-strand template for the mutagenic oligonucleotide below:

5'- CCC ATT AAG ATT <u>GGC CGC CTG GGC C</u>GA CAT GTT GCT GG - 3'   (SEQ ID NO:9)

20

This primer introduced an SfiI site (indicated by underlining) which allowed correct forms to be screened for by the presence of this unique restriction site. Subcloning the EcoRI-SstII fragment back into the pBLapr vector gave a version of the plasmid containing an SfiI site.

Plasmid pHP13 (Haima et al., Mol. Gen. Genet., Vol. 209, pp. 335-342 (1987)) (Figure 5)

25   was digested with restriction enzymes EcoRI and HindIII and the resulting vector purified on a polyacrymide gel and then eluted. Plasmid pBLapr was digested with HindIII, Asp718 and in a separate incubation with Asp718, EcoRI and gel purified. Two bands, HindIII-Asp718 (1203 bp) and Asp718-EcoRI (1253 bp)were gel purified, eluted from the gel and ligated into the vector by a 3-way ligation, to give plasmid pHP.BL, the plasmid used in expression of the α-amylase (Figure

30   7).

EXAMPLE 2

Construction Of Plasmid Encoding α-Amylase
Comprising Substitutions At E119C/S130C Or D124C/R127C

A pBLapr plasmid having threonine substituted for methionine at amino acid 15 was

35   constructed according to U.S. Patent Application Serial No. 08/194,664 (PCT Publication No. WO 94/18314). To introduce the additional cysteine residues, the following mutagenic primers encoding for substitutions of E119C/S130C and D124C/R127C were used together with non-mutagenic primers to introduce the desired mutations into linear multiple tandem repeats of the plasmid by the method of multimerization as described below.

_119C/130C_
AA CCg Cgg TTT gCg TCg ATC CCg CTg ACC gCA ACC gCg TAA TTT gCg gAg AAC ACC (SEQ ID NO:10)

5   _124C/127C_
TCg ATC CCg CTT gCC gCA ACTgCg TAA TTT CAg gAg AA (SEQ ID NO:11)

A fragment starting at the mutagenic primer (shown above) and ending at the 3' end of the coding region was generated by PCR. This fragment was gel purified and used to generate long,

10   linear tandem repeats of the plasmid encoding the desired cysteine mutations as follows:

The vector (pBLapr/M15T) was linearized by restriction digest (Sal I) and purified using Qiagen kits. The multimerization reactions typically contained 5.4 mM Tris buffer pH 8.0, 1x XL buffer (Perkin Elmer, Branchburg, NJ), 0.2 mM dNTPs, 1.1 mM Mg(OAc)$_2$, 3 ng/μl incoming fragment, 0.15 ng/μl linearized vector, 4 U _rTth_ DNA polymerase, XL (Perkin Elmer) in 100 μl

15   reaction mixture. PCR reactions were typically performed in a thermocycler under the following conditions: 20 cycles (15s 94°C, 5 min 68°C) and 15 cycles (15s 94°C, 10 min 68°C).

The resulting multimers were transformed directly into _B. subtilis_ competent cells using standard techniques. Plasmid DNA was isolated from the transformants using standard techniques.

20   Mutations were confirmed by dideoxy sequencing (Sanger et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 74, pp. 5463-5467 (1977)).

EXAMPLE 3

Transformation Of Plasmids Into _Bacillus subtilis_,
25   ### Expression And Purification of Mutant α-Amylase

α-Amylase may be expressed in _Bacillus subtilis_ after transformation with the plasmids described above. pHP13 is a plasmid able to replicate in _E. coli_ and in _Bacillus subtilis_. Plasmids containing different variants were constructed using _E. coli_ strain MM294, the plasmids isolated and then transformed into _Bacillus subtilis_ as described in Anagnostopoulos et al., J. Bacter., Vol.

30   81, pp. 741-746 (1961). The _Bacillus_ strain had been deleted for two proteases (Δapr, Δnpr) (see e.g., Ferrari et al., U.S. Patent No. 5,264,366) and for amylase (ΔamyE) (see e.g., Stahl et al., J. Bacter., Vol. 158, pp. 411-418 (1984)). After transformation, the sacU(Hy) mutation (Henner et al., J. Bacter., Vol., 170, pp. 296-300 (1988)) was introduced by PBS-1 mediated transduction (Hoch, J. Bact., Vol. 154, pp. 1513-1515 (1983)).

35   Secreted amylase was recovered from _Bacillus subtilis_ cultures as follows: Sodium chloride was added to the culture supernatant to 20mM and the pH was adjusted to approximately 7.0 with 1M tris buffer, pH 7.2. The supernatant was then heated to 70°C for 15 minutes, and the precipitate removed by centrifugation. Ammonium sulphate was added the supernatant to 1.3M followed by 20ml phenyl sepharose fast flow 6 (high substitution) resin (Pharmacia). Following

40   agitation, resin was separated by filtration, and washed in 1M ammonium sulphate, 20mM

ammonium acetate pH 7.0, 5mM calcium chloride. The bound amylase was eluted into 20mM ammonium acetate pH 7.0, 5mM calcium chloride, and precipated by addition of ammonium sulphate to 70% saturation. The precipated material was pelleted by centrifugation, redissolved in a minimum volume of 20mM ammonium acetate pH 7.0, 5mM calcium chloride and dialysed

5   against the same buffer.

Concentration was determined using the soluble substrate assay, assuming wild-type specific activity.

EXAMPLE 4
Assay For Determining α-Amylase Activity

10   Soluble Substrate Assay: A rate assay was developed based on an end-point assay kit supplied by Megazyme (Aust.) Pty. Ltd. A vial of substrate (p-nitrophenyl maltoheptaoside, BPNPG7) was dissolved in 10ml of sterile water followed by a 1:4 dilution in assay buffer (50mM maleate buffer, pH 6.7, 5mM calcium chloride, 0.002% Tween20). Assays were performed by

15   adding 10μl of amylase to 790μl of the substrate in a cuvette at 25°C. Rates of hydrolysis were measured as the rate of change of absorbance at 410nm, after a delay of 75 seconds. The assay was linear up to rates of 0.2 absorption units/min.

α-Amylase protein concentration was measured using the standard Bio-Rad Assay (Bio-Rad Laboratories) based on the method of Bradford, Anal. Biochem., Vol. 72, p. 248 (1976) using

20   bovine serum albumin standards.

EXAMPLE 5
Preparation and Testing of Additional
Mutant Alpha-Amylases for Thermal Stability

25   Mutant B. licheniformis alpha-amylases were prepared having substitutions at M15T or M15T/E119C/S130C. Thermal inactivation rates for the various mutants were measured according to the following procedure. Amylase stock solutions were dialysed extensively into 20 mM ammonium acetate, 4 mM CaCl₂ pH 6.5. Each sample was split into two equal vials and dithiothreitol added to one of the vials at 10 mM and stored at least overnight at 4°C. For

30   measurement of stability, this stock was diluted >50fold into 50mM ammonium acetate, 5mM CaCl₂, 0.02% Tween 20 pH 4.8 to a final concentration of between 30 and 50 μg/ml. For those stocks containing 10mM DTT, the dilution buffers contained 1mM DTT. Six 100μl aliquots were put into eppendorf tubes and placed into a water bath or hot block at 83˙C. The eppendorf tubes were removed at regular, measured intervals of between 30 seconds and 5 minutes and placed on

35   ice to stop the inactivation. The residual activity was assayed using a soluble substrate as described in Example 4. The natural log of the activity was plotted against time of incubation, and the rate constant for inactivation obtained from the slope of the straight line. Results for various mutants are provided in Table 1.

TABLE 1

Amylase Mutant	Temperature	Inactivation Rate Constant k(min⁻¹)	Half Life (minutes)
M15T/E119C/S130C (+DTT)	63.9	0.0278	24.932
M15T/E119C/S130C (+DTT)	65	0.0461	25.035
M15T/E119C/S130C (+DTT)	66.2	0.0632	10.967
M15T/E119C/S130C (+DTT)	67.4	0.0884	7.840
M15T/E119C/S130C (+DTT)	68.5	0.102	6.795
M15T/E119C/S130C (+DTT)	69.5	0.124	5.590
M15T/E119C/S130C (+DTT)	70.4	0.168	4.126
M15T/E119C/S130C (+DTT)	71.3	0.202	3.431
M15T/E119C/S130C (+DTT)	74.2	0.67	1.034
M15T/E119C/S130C (+DTT)	74.2	0.682	1.016
M15T/E119C/S130C (-DTT)	67.5	0.0481	14.410
M15T/E119C/S130C (-DTT)	69	0.0728	9.521
M15T/E119C/S130C (-DTT)	70.2	0.0978	7.087
M15T/E119C/S130C (-DTT)	71.4	0.145	4.780
M15T/E119C/S130C (-DTT)	72.5	0.186	3.726
M15T/E119C/S130C (-DTT)	73.4	0.254	2.729
M15T/E119C/S130C (-DTT)	74.5	0.305	2.272
M15T/E119C/S130C (-DTT)	75.5	0.4	1.733
M15T/E119C/S130C (-DTT)	72.9	0.158	4.387
M15T/E119C/S130C (-DTT)	72.9	0.156	4.443
M15T/E119C/S130C (-DTT)	72.9	0.146	4.747
M15T	67.5	0.051	13.590
M15T	69	0.082	8.452
M15T	70.2	0.112	6.188
M15T	71.4	0.143	4.847
M15T	72.5	0.191	3.629
M15T	72.9	0.199	3.483
M15T	72.9	0.208	3.332
M15T	72.9	0.244	2.841
M15T	73.4	0.208	3.332
M15T	74.5	0.302	2.295
M15T	75.5	0.421	1.646

As shown in Table 1, mutant enzymes having introduced therein two cysteine residues capable of forming a disulfide bond showed significantly increased stability over the mutant M15T

enzyme with no introduces cysteine bonds. Additionally, as shown in Table 1, mutant enzymes having introduced therein a disulfide bond between E119C and S130C showed significantly improved stability over the M15T mutant or the M15T/E119C/S130C mutant which was treated with DTT (i.e., disulfide bond reduced and/or broken).

WE CLAIM:

1. A mutant α-amylase capable of forming a disulfide bond between a first and a second cysteine residue, wherein said mutant α-amylase is derived from a precursor α-amylase and said first cysteine residue results from a substitution or addition of an amino acid to said precursor α-amylase.

2. The mutant α-amylase according to claim 1, wherein said first cysteine residue corresponds to a residue in said precursor α-amylase having a Cα-Cα bond distance of between about 4.4-6.8 Angstroms and a Cβ-Cβ bond distance of between about 3.45 and 4.5 Angstroms with said second cysteine residue or a residue corresponding to said second cysteine residue in said precursor α-amylase.

3. The α-amylase according to claim 2, wherein said α-amylase comprises a substitution corresponding to E119C/S130C and/or D124C/R127C Bacillus licheniformis.

4. The α-amylase according to claim 1, wherein said α-amylase is derived from a bacterial or fungal source.

5. The α-amylase according to claim 1, wherein said α-amylase is derived from Bacillus.

6. The α-amylase according to claim 5, wherein said α-amylase is derived from Bacillus licheniformis, Bacillus stearothermophilus or Bacillus amyloliquefaciens.

7. The α-amylase according to claim 1 wherein said α-amylase further comprises the deletion or substitution of a residue corresponding to M15, A33, A52, S85, N96, V129, H133, S148N, S187, N188, A209, A269 and/or A379 in Bacillus licheniformis.

8. An α-amylase that is the expression product of a mutated DNA sequence encoding an α-amylase, the mutated DNA sequence being derived from a precursor α-amylase by a substitution corresponding to M15T/E119C/S130C/N188S, M15L/E119C/S130C/N188S, M15T/E119C/S130C/H133Y/N188S, M15T/E119C/S130C/H133Y/N188S/A209V, M15T/E119C/S130C/N188S/A209V, M15T/E119C/V128E/S130C/H133Y/N188S, M15T/E119C/S130C/S187D/N188S, M15T/E119C/S130C/H133Y/N1888S/A209V, M15T/E119C/S130C/H133Y/S148N/N188S/A209V/A379S, or M15T/E119C/S130C/H133Y in Bacillus licheniformis.

9. The α-amylase according to claim 1, wherein substitution further comprises substituting or deleting a residue corresponding to M15T, W138Y and/or M197T in *Bacillus licheniformis*.

10. A DNA encoding the α-amylase according to claim 1.

11. An expression vector comprising the DNA of claim 10.

12. A host cell transformed with the expression vector of claim 11.

13. An α-amylase according to claims 1, 3 or 7 having enhanced low pH performance.

14. A detergent composition comprising the α-amylase according to claim 1.

15. The detergent composition according to claim 14 , wherein said detergent is useful for cleaning soiled laundry and/or soiled dishes.

16. A method of liquefying starch comprising contacting a slurry of starch with the α-amylase according to claim 1.

148N

119-130

124-127
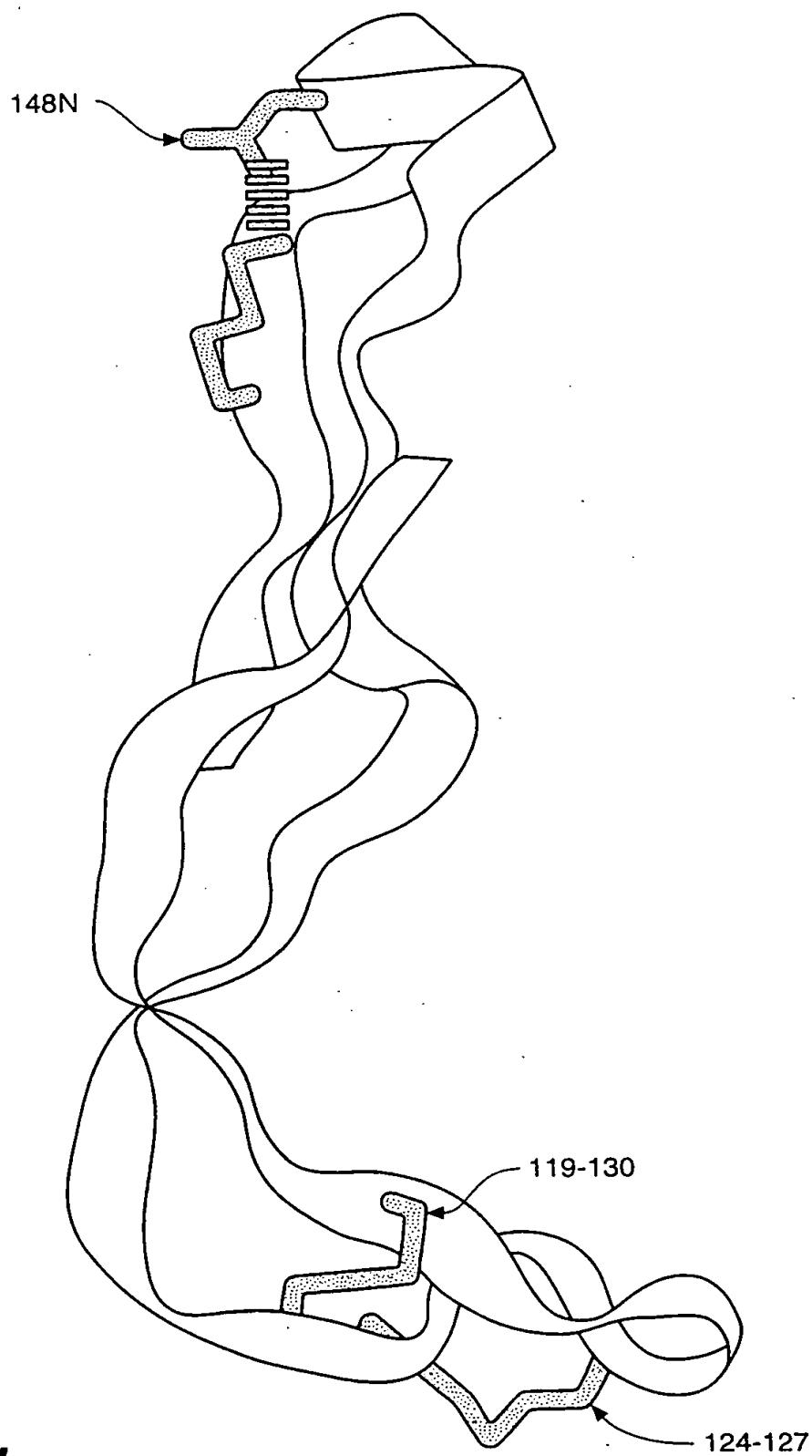
FIG._1

```
          10                        30                        50
AGCTTGAAGAAGTGAAGAAGCAGAGAGGCTATTGAATAAATGAGTAGAAAGCGCCATATC


          70                        90                        110
GGCGCTTTTCTTTTGGAAGAAAATATAGGGAAAATGGTACTTGTTAAAAATTCGGAATAT


          130                       150                       170
TTATACAACATCATATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACG
                                                   M  K  Q  Q  K  R

          190                       210                       230
GCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTTGCTGCCTCATTCTGC
  L  Y  A  R  L  L  T  L  L  F  A  L  I  F  L  L  P  H  S  A

          250                       270                       290
AGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAA
  A  A  A  A  N  L  N  G  T  L  M  Q  Y  F  E  W  Y  M  P  N

          310                       330                       350
TGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTAT
  D  G  Q  H  W  K  R  L  Q  N  D  S  A  Y  L  A  E  H  G  I

          370                       390                       410
TACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGG
  T  A  V  W  I  P  P  A  Y  K  G  T  S  Q  A  D  V  G  Y  G

          430                       450                       470
TGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTA
  A  Y  D  L  Y  D  L  G  E  F  H  Q  K  G  T  V  R  T  K  Y

          490                       510                       530
CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGT
  G  T  K  G  E  L  Q  S  A  I  K  S  L  H  S  R  D  I  N  V

          550                       570                       590
TTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGC
  Y  G  D  V  V  I  N  H  K  G  G  A  D  A  T  E  D  V  T  A

          610                       630                       650
GGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGC
  V  E  V  D  P  A  D  R  N  R  V  I  S  G  E  H  L  I  K  A

          670                       690                       710
CTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTG
  W  T  H  F  H  F  P  G  R  G  S  T  Y  S  D  F  K  W  H  W

          730                       750                       770
GTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTT
  Y  H  F  D  G  T  D  W  D  E  S  R  K  L  N  R  I  Y  K  F

          790                       810                       830
TCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGAT
  Q  G  K  A  W  D  W  E  V  S  N  E  N  G  N  Y  D  Y  L  M
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FIG._2A

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                850                          870                          890
GTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCAC
  Y  A  D  I  D  Y  D  H  P  D  V  A  A  E  I  K  R  W  G  T
                910                          930                          950
TTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAA
  W  Y  A  N  E  L  Q  L  D  G  F  R  L  D  A  V  K  H  I  K
                970                          990                         1010
ATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTT
  F  S  F  L  R  D  W  V  N  H  V  R  E  K  T  G  K  E  M  F
               1030                         1050                         1070
TACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAAC
  T  V  A  E  Y  W  Q  N  D  L  G  A  L  E  N  Y  L  N  K  T
               1090                         1110                         1130
AAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGAC
  N  F  N  H  S  V  F  D  V  P  L  H  Y  Q  F  H  A  A  S  T
               1150                         1170                         1190
ACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCC
  Q  G  G  G  Y  D  M  R  K  L  L  N  G  T  V  V  S  K  H  P
               1210                         1230                         1250
GTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGGCAATCGCTTGAGTC
  L  K  S  V  T  F  V  D  N  H  D  T  Q  P  G  Q  S  L  E  S
               1270                         1290                         1310
GACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGG
  T  V  Q  T  W  F  K  P  L  A  Y  A  F  I  L  T  R  E  S  G
               1330                         1350                         1370
ATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAAT
  Y  P  Q  V  F  Y  G  D  M  Y  G  T  K  G  D  S  Q  R  E  I
               1390                         1410                         1430
TCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGG
  P  A  L  K  H  K  I  E  P  I  L  K  A  R  K  Q  Y  A  Y  G
               1450                         1470                         1490
AGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAG
  A  Q  H  D  Y  F  D  H  H  D  I  V  G  W  T  R  E  G  D  S
               1510                         1530                         1550
CTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCG
  S  V  A  N  S  G  L  A  A  L  I  T  D  G  P  G  G  A  K  R
               1570                         1590                         1610
AATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTC
  M  Y  V  G  R  Q  N  A  G  E  T  W  H  D  I  T  G  N  R  S
               1630                         1650                         1670
GGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGT
  E  P  V  V  I  N  S  E  G  W  G  E  F  H  V  N  G  G  S  V
```

1690   1710   1730
TTCAATTTATGTTCAAAGATAGAAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTT
 S   I   Y   V   Q   R   *
1750   1770   1790
TTTTTATTTTGCCCGTCTTATAAATTTCTTTGATTACATTTTATAATTAATTTTAACAAA

1810   1830   1850
GTGTCATCAGCCCTCAGGAAGGACTTGCTGACAGTTTGAATCGCATAGGTAAGGCGGGGA

1870   1890   1910
TGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAATGTGTCGAAAATGACGGTATC

1930   1950
GCGGGTGATCAATCATCCTGAGACTGTGACGGATGAATTGAAAAAGCT

FIG._2C

FIG._2

FIG._2A

FIG._2B

FIG._2C
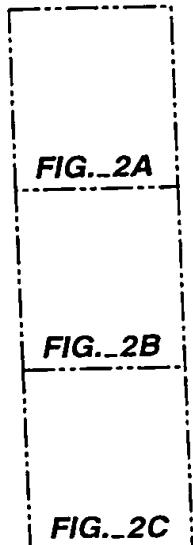
```
          10                          30                      50
ANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD


          70                          90                     110
LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV


         130                         150                     170
DPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGK


         190                         210                     230
AWDWEVSNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF


         250                         270                     290
LRDWVNHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGG


         310                         330                     350
GYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQ


         370                         390                     410
VFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVA


         430                         450                     470
NSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIY


VQR
```

FIG._3

```
                 Am-Lich = B.Licheniformis    Am-Amylo = B.amyloliquefaciens    Am-Stearo = B.stearothermophilus

                 1                                                                 1
Am-Lich    1     ......MKQQ KRLYARLLTL LFALIFLLPH .......... ....SAAA   AANLNGTLMQ YFEWYMPNDG        19
Am-Amylo         MRGRNMIQK  RKRTVSFRLV LMCTLLFVSL ......PITK           TSAVNGTLMQ YFEWYTPNDG        60
Am-Stearo        ......VLTF HRIIRKGWMF LLAFLLTASL FCPTGRHAKA           AAPFNGTMMQ YFEWYLPDDG

Am-Lich    61    QHWKRLQNDS AYLAEHGITA VWIPPAYKGT SQADVGYGAY DLYDLGEFHQ KGTVRTKYGT                  79
Am-Amylo         QHWKRLQNDA EHLSDIGITA VWIPPAYKGL SQSDNGYGPY DLYDLGEFQQ KGTVRTKYGT                  120
Am-Stearo        TLWTKVANEA NNLSSLGITA LSLPPAYKGT SRSDVGYGVY DLYDLGEFNQ KGTVRTKYGT

Am-Lich    121   KGELQSAIKS LHSRDINVYG DVVINHKGGA DATEDVTAVE VDPADRNRVI SGEHLIKAWT                  139
Am-Amylo         KSELQDAIGS LHSRNVQVYG DVVLNHKAGA DATEDVTAVE VNPANRNQET SEEYQIKAWT                  180
Am-Stearo        KAQYLQAIQA AHAAGMQVYA DVVFDHKGGA DGTEWWDAVE VNPSDRNQEI SGTYQIQAWT

Am-Lich    181   HFHFPGRGST YSDFKWHWYH FDGTDWDESR KLNRIYKF... QGKAWDWEVS NENGNYDYLM                  197
Am-Amylo         DFRFPGRGNT YSDFKWHWYH FDGADWDESR KISRIFKFRG EGKAWDWEVS SENGNYDYLM                  240
Am-Stearo        KFDFPGRGNT YSSFKWRWYH FDGVDWDESR KLSRIYKFRG IGKAWDWEVD TENGNYDYLM

Am-Lich    241   YADIDYDHPD VAAEIKRWGT WYANELQLDG FRLDAVKHIK FSFLRDWVNH VREKTGKEMF                  257
Am-Amylo         YADVDYDHPD VVAETKKWGI WYANELSLDG FRIDAAKHIK FSFLRDWVQA VRQATGKEMF                  300
Am-Stearo        YADLDMDHPE VVTELKNWGK WYVNTTNIDG FRLDGLKHIK FSFFPDWLSY VRSQTGKPLF

Am-Lich    301   TVAEYWQNDL GALENYLNKT NFNHSVFDVP LHYQFHAAST QGGGYDMRKL LNGTVVSKHP                  317
Am-Amylo         TVAEYWQNNA GKLENYLNKT SFNQSVFDVP LHFNLQAASS QGGGYDMRRL LDGTVVSRHP                  360
Am-Stearo        TVGEYWSYDI NKLHNYITKT NGTMSLFDAP LHNKFYTASK SGGAFDMRTL MTNTLMKDQP
```

FIG._4A

```
                                                                        377
                                                                        420
361
Am-Lich     LKSVTFVDNH DTQPGQSLES TVQTWFKPLA YAFILTRESG YPQVFYGDMY GTKGDSQREI
Am-Amylo    EKAVTFVENH DTQPGQSLES TVQTWFKPLA YAFILTRESG YPQVFYGDMY GTKGTSPKEI
Am-Stearo   TLAVTFVDNH DTNPAKR.CS HGRPWFKPLA YAFILTRQEG YPCVFYGDYY GI......PQYNI

                                                                        437
                                                                        480
421
Am-Lich     PALKHKIEPI LKARKQYAYG AQHDYFDHHD IVGWTREGDS SVANSGLAAL ITDGPGGAKR
Am-Amylo    PSLKDNIEPI LKARKEYAYG PQHDYIDHPD VIGWTREGDS SAAKSGLAAL ITDGPGGSKR
Am-Stearo   PSLKSKIDPL LIARRDYAYG TQHDYLDHSD IIGWTREGVT EKPGSGLAAL ITDGAGRSKW

                                                               483      540
481
Am-Lich     MYVGRQNAGE TWHDITGNRS EPVVINSEGW GEFHVNGGSV SIYVQR......
Am-Amylo    MYAGLKNAGE TWYDITGNRS DTVKIGSDGW GEFHVNDGSV SIYVQK......
Am-Stearo   MYVGKQHAGK VFYDLTGNRS DTVTINSDGW GEFKVNGGSV SVWVPRKTTV STIARPITTR

                                                                        559
541
Am-Lich     ............
Am-Amylo    ............
Am-Stearo   PWTGEFVRWH EPRLVAWP*
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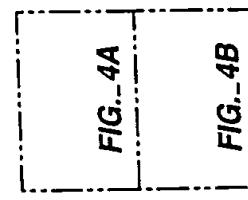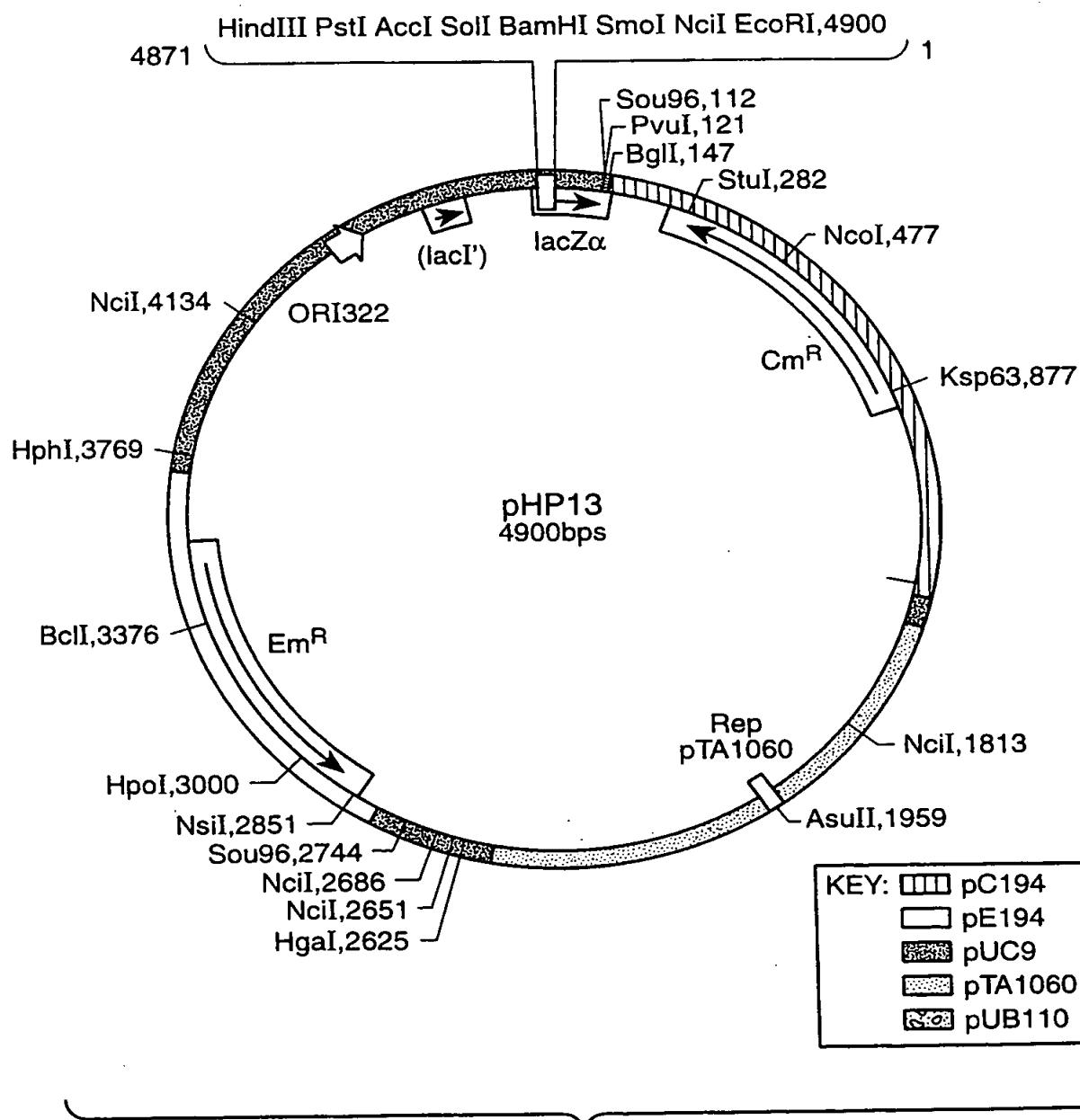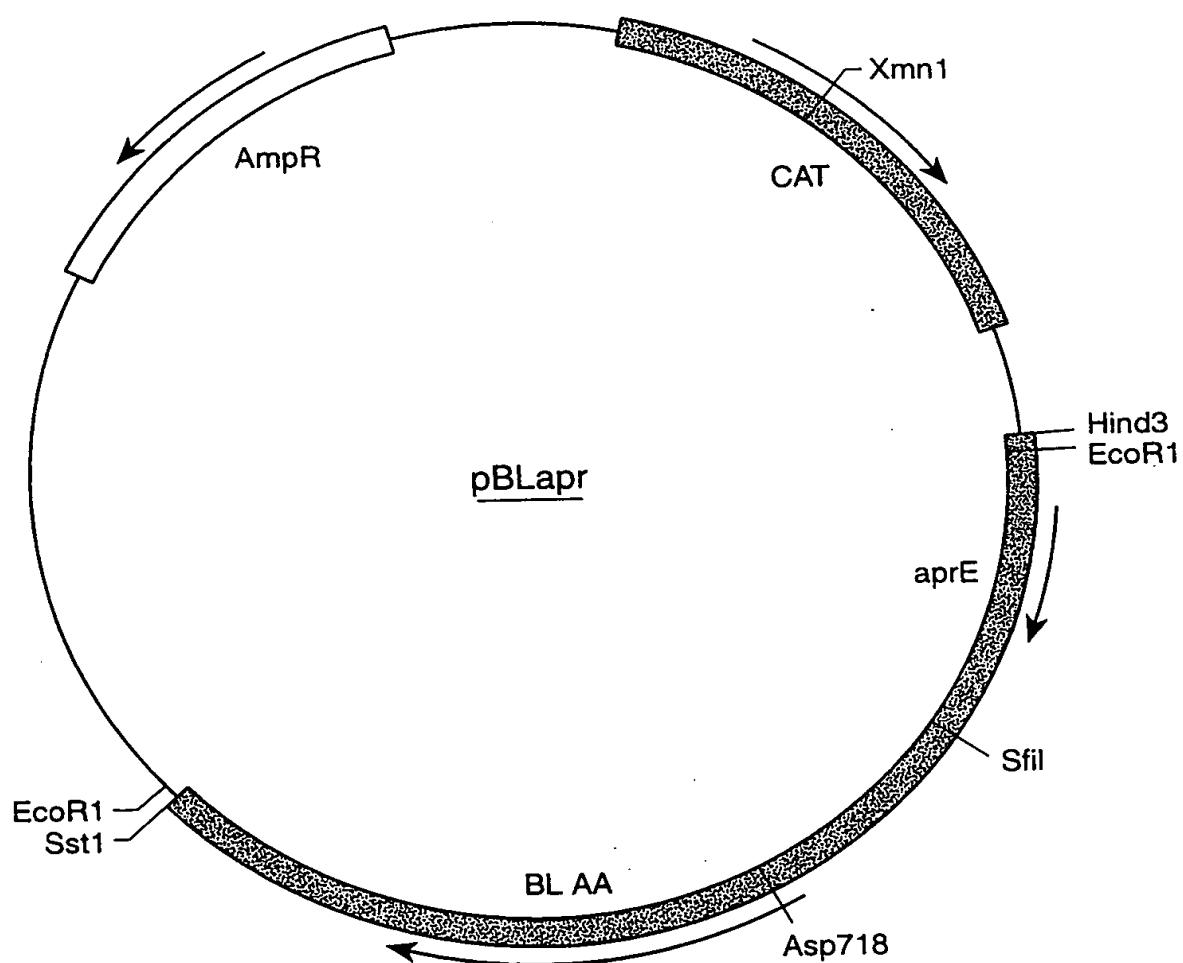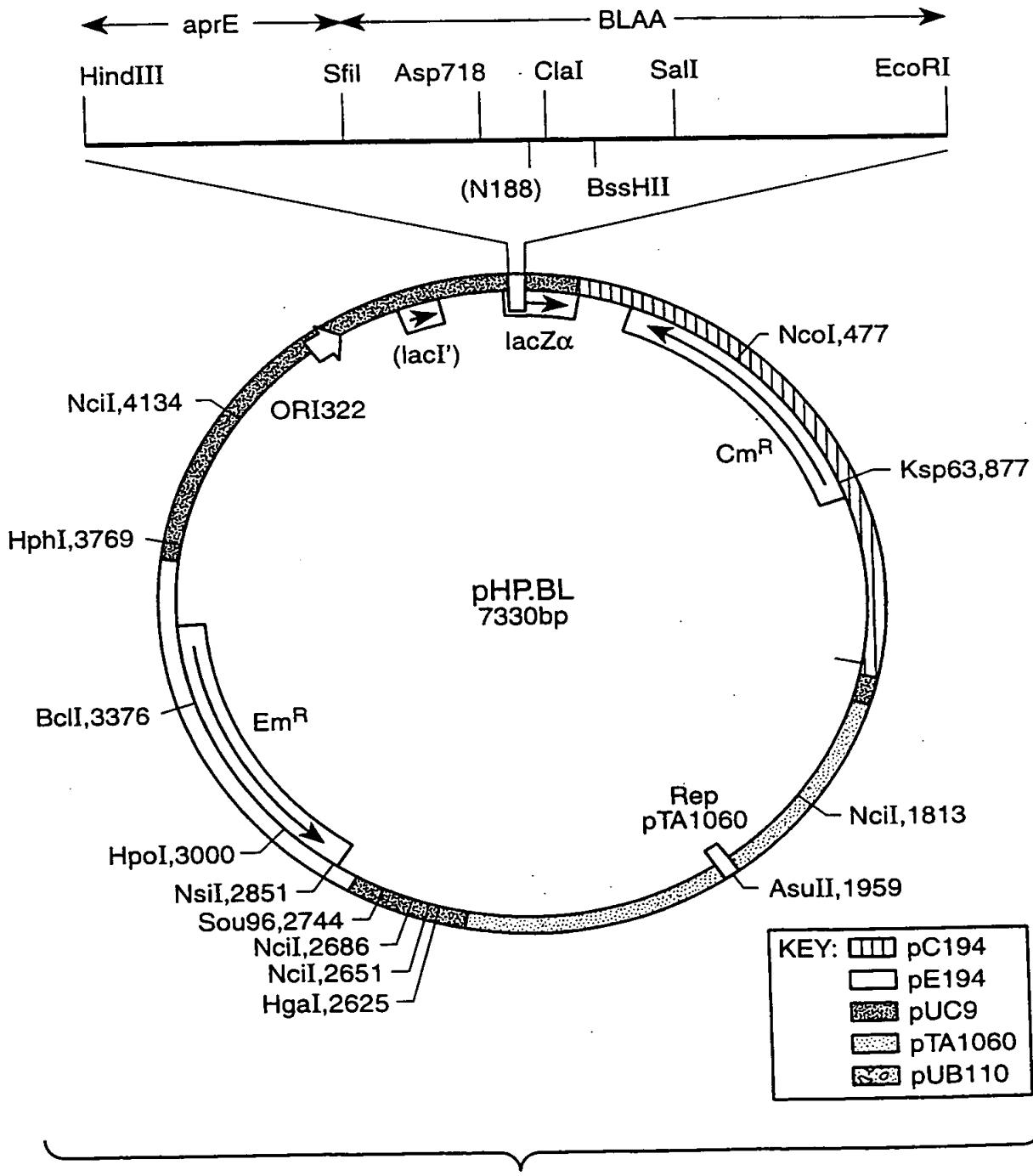
FIG._4B

FIG._4A

FIG._4B

FIG._4

FIG._5

FIG._6

FIG._7

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/55   C12N9/28   C12P19/14   C12S3/12   C11D3/386
         C12N1/21   //(C12N9/28,C12R1:10)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C12P   C11D   C12S

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOWDHAMINI R ET AL: "Stereochemical modeling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis." PROTEIN ENGINEERING, vol. 3, no. 2, 1989, pages 95-103, XP002085412 cited in the application see abstract see table VI see page 96, column 1, line 4 – page 97, column 1, line 23 ———	1,2

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
23 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
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A	WO 96 39528 A (GENENCOR INT ;MITCHINSON COLIN (GB); REQUADT CAROL (US); ROPP TRAC) 12 December 1996 see claims ---	7-9
A	WO 96 30481 A (GENENCOR INT) 3 October 1996 see claims ---	7-9
A	WO 95 35382 A (GIST BROCADES BV ;LAAN JAN METSKE V D (NL); AEHLE WOLFGANG (NL)) 28 December 1995 cited in the application see claims 1-4 ---	7,8
A	FR 2 665 178 A (AGRONOMIQUE INST NAT RECH) 31 January 1992 cited in the application see abstract ---	7,8
A	WO 95 10603 A (BORCHERT TORBEN VEDEL ;THELLERSEN MARIANNE (DK); ZEE PIA V D (DK);) 20 April 1995 cited in the application see claim 17 -----	7,8

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WO 9630481	A	03-10-1996	AU BR EP NO	5322696 A 9607751 A 0815193 A 974402 A	16-10-1996 23-06-1998 07-01-1998 19-11-1997
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